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Enduring Cognitive, Emotional and Neurogenic Alterations Induced by Alcohol and Methamphetamine Exposure in Adolescent Rats

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A thesis submitted in partial fulfilment of the

Requirements for the degree of

Master of Science in Psychology

By

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Abbreviations

BrdU	2,5-bromodeoxyuridine
DCX	Doublecortin
DG	Dentate Gyrus
ETOH	Ethanol
EPM	Elevated Plus Maze
MA	Methamphetamine
OF	Open Field
PBS	Phosphate buffered saline
PFC	Paraformaldehyde
PND	Post-Natal Days
RAM	Radial Arm Maze
RME	Reference Memory Errors
RM	Reference Memory
Sal	Saline
Tx	Triton X-100
WM	Working Memory
WME	Working Memory Errors

Abstract

A high proportion of young methamphetamine (MA) users simultaneously consume alcohol. However, the potential neurological and behavioural alterations induced by such a drug combination have not been systematically examined. Here, we studied in adolescent rats the long-term effects of alcohol, MA, and an alcohol-MA combination on anxiety-like behaviour, memory, and neurogenesis in the adult hippocampus. Rats received saline (control), ethanol (ETOH, 1.5 g/kg), MA (MA, 2 mg/kg), or ethanol and MA combined (ETHOH-MA, 1.5 g/kg ethanol plus 2 mg/kg MA) via oral gavage, once daily for 5 consecutive days. Open field (OF), elevated plus maze (EPM) and radial arm maze (RAM) tests were conducted following a 15-day withdrawal period. The results showed alterations in exploratory behaviour in the OF in the MA and ETOH-MA groups, and anxiety-like effects in the EPM in all three drug treatment groups. Further, all three drug groups exhibited reference memory deficits in the RAM, but only the combination treatment group displayed alterations in working memory performance. Neurogenesis was assessed by measuring the number of doublecortin(DCX)-void gaps, the length of such gaps and the pattern of arborisation of DCX+ neurons in the dentate gyrus (DG). Both MA and ETOH-MA treatments increased the length of DCX-void gaps in the DG but only ETOH-MA treatment increased the number of such gaps. An increased occurrence and length of the DCX-void gaps correlated with decreased exploratory activity in the OF, and impaired working memory in the RAM was associated with an augmented number of gaps. These findings suggest that persistent behavioural deficits are linked to the alterations in adult hippocampal neurogenesis produced by alcohol and MA exposure, and highlight the potential mental health risks associated with the simultaneous use of both substances.

Introduction

1.1 Methamphetamine and Alcohol

Methamphetamine is a psychostimulant drug with powerful reinforcing properties and is the second most abused illicit drug in the world (UNODC, 2013). In Australia and New Zealand, it is commonly referred to as 'P' or 'ice' depending on its form and method of delivery. Methamphetamine can increase wakefulness, improve reactions and attention as well as improve performance on demanding mental tasks and was commonly used in world war two for pilots flying long distances during the pacific front. After the war, government stockpiles were made available and the pacific/oceanic region experienced its first methamphetamine abuse problems in the early 50's (Toolaney, 2007). Troublingly, the same region is now experiencing a revival of the methamphetamine crisis at an epidemic level. Reports have shown that instances of smoking crystal methamphetamine are on the rise in the oceanic region, especially in Australia and New Zealand, and seizures amongst the Asia and south pacific region are at their highest level in five years (UNODC, 2013).

Increased methamphetamine use raises another issue, in methamphetamine users the simultaneous or concurrent use of one or more psychoactive drugs is common. Users of methamphetamine were significantly more likely to co-abuse other substances in a rave-type setting including legal 'party drugs', ecstasy, and alcohol (Kinner & Degenhardt, 2008). Alcohol is frequently abused worldwide and is often co-abused drug with methamphetamine and individuals who had daily episodes of alcohol intoxication were estimated to be five times more likely to have recently smoked methamphetamine when compared to non-drinkers (Furr et al, 1999).

Alcohol is the most commonly abused substance worldwide, illegal or otherwise, particularly in countries that adopt a binge drinking culture (UNODC 2015). For both methamphetamine and alcohol, the first instance of use usually occurs during adolescence which is a period of

critical brain development and is known for risky decision making and behaviour. New Zealand's teenage alcohol use is high even by international standards, while approximately 2.3% of adolescents aged between 15 and 64 in New Zealand have admitted to using it on a regular basis (UNODC, World drug report 2013, United Nations).

1.2 Poly-Drug Abuse

Patterns of poly-drug use are likely a better representation of drug taking behaviour and in this study we aim to address the combination of the world's most popular intoxicant (through its active compound ethanol) and the popular illicit drug, methamphetamine. It has been shown that methamphetamine users are also moderate to heavy users of alcohol and conversely that adolescents who drank alcohol were often characterized through the use of illegal substances, especially methamphetamine (O'Grady et al, 2008; Brecht et al, 2008). Current studies into the neuroscience of addiction are increasingly recognizing the role and challenges that polydrug use (the concurrent or simultaneous use of more than one substance over a given period) creates in the study of individuals with addictions and animal models of drug abuse (Schensul et al, 2005). This instance of polydrug use is particularly concerning because the combination appears to be deadly with ethanol present in about a third of methamphetamine-related deaths (Medenlson et al, 1995).

Research into alcohol and methamphetamine's effects on adolescent cognition and behaviour have been thoroughly examined in previous studies but almost exclusively independent of one another and very little is known about the cognitive and behavioural effects of the two substances used together (Toolaney, 2007). One recent study found that co-administration of methamphetamine and ethanol produce an observable anxiogenic effect in the elevated plus maze and it was suggested that this could have been caused by neurotoxicity in the dentate gyrus of the hippocampus (Chuang et al, 2011). This is important as the hippocampus

undergoes changes through the process of neurogenesis which incorporates maturing neurons into the dentate gyrus and a disruption in neurogenesis here caused by drug exposure could be responsible for deficits in cognition and alterations in behaviour.

Many studies have identified that the polydrug use, especially among adolescents and young adults, has a unique series of consequences during a time which is critical for brain development linking them to a greater risk of negative health outcomes as well as deficits in cognition and social behaviour (Collins et al 1999). Long term abuse of drugs is known to impair normal brain development during adolescence by interrupting important stages of neurodevelopment including changes in plasticity and neuronal connectivity making adolescents more vulnerable compared to adults with many developing cognitive deficits (especially learning and memory) that persist into adulthood (Guerri & Pascual, 2010).

These persistent cognitive deficits in memory and learning caused by drug exposure may implicate an interference with information processing abilities of the hippocampal formation, an area of the brain well known for its' role in different forms of cognitive functioning including memory and emotional learning (Chuang et al, 2010). Importantly, it is the hippocampus's ability to self-regenerate and renew that becomes disrupted during long term drug abuse (Canales, 2007; 2012). This process of neurogenesis, where adult-born neurons are incorporated into the dentate gyrus of the hippocampal formation, may be an important mechanism in the encoding, consolidation and inter-region transfer of information beyond the hippocampal network. Alcohol and drugs of abuse interfere with the process of neurogenesis by causing decreases in neural stem proliferation and new-born cell survival (Morris et al, 2010) impeding the incorporation and/or development of these new cells.

Considerable evidence suggests that stimulant intoxication with MA is associated with excessive drinking in adolescents and young adults (McKetin et al. 2014; Olthuis et al. 2013).

Such pattern of poly-substance abuse is not only linked to increased risk of negative health outcomes, including neurotoxicity, substance abuse progression, cognitive impairment and psychiatric vulnerability, but also complicates the implementation of effective intervention programmes (Barrett et al. 2006; Grov et al. 2009). Albeit the mechanisms mediating the physiological interactions between alcohol and MA are poorly understood, the combined use of both substances induces heightened peripheral physiological effects, reduces MA-induced sleep disturbances and increases perceived global intoxication ratings (Kirkpatrick et al. 2012; Mendelson et al. 1995), which may partly account for the popularity of their simultaneous use.

1.3 Neurogenesis

Neurogenesis in the adult hippocampus is a key physiological process with wide implications for understanding neural plasticity, functional modifiability, normal behaviour and cognition, and psychopathology (Cameron and Glover 2015; Canales 2010; Drew et al. 2013; Opendak and Gould 2015). Addictive drugs, including opiates, alcohol and psychomotor stimulants, such as MA, impair adult neurogenesis in the hippocampus, disrupting the proliferation of neural progenitors, neuronal survival rates and maturation and differentiation in a drug and regimen-specific fashion (Canales 2013; Mandyam and Koob 2012). Converging evidence revealed that alcohol exposure produces severe alterations of neurogenic processes in the adult hippocampus. Semi-chronic binge exposure to gavaged alcohol decreased proliferation and survival of new-born cells in the dentate gyrus (DG) of rats (Nixon and Crews 2002), with alcohol self-administration producing similar effects, including inhibition of proliferating nuclear antigen expression, decreased survival of 2,5-bromodeoxyuridine (BrdU) labelling, and reduced size of the dendritic arbours of doublecortin-labelled, immature differentiating neurons (He et al. 2005). Similarly, short and long daily access to

MA self-administration decreased proliferation, early phase maturation and survival of adult-born hippocampal neurons (Mandyam et al. 2008).

Impairments in adult hippocampal neurogenesis, especially those evoked by psychoactive substances, have been consistently linked to deficits in learning and memory function and dysregulation of affective behaviour (Canales 2010), craving and perpetuation of addictive behaviours (Mandyam and Koob 2012), and neuropsychiatric vulnerability (DeCarolis and Eisch 2010). Previous research showed that co-administration of alcohol and the psychedelic stimulant, 3,4-methylenedioxy-methamphetamine (MDMA or Ecstasy), at doses that did not produce apparent cognitive impairments when given separately, produced persistent memory deficits as well as granule cell depletion and alterations in hippocampal neurogenesis in adolescent rats (Hernandez-Rabaza et al. 2010). Similar mutually potentiating effects of alcohol and MDMA have been reported in rats exposed prenatally, with neurogenic and behavioural deficits induced by the combination extending into adulthood (Canales and Ferrer-Donato 2014). Despite the prevalent use of both alcohol and MA at a young age in humans, the potential deleterious effects of such drug combination on adult hippocampal neurogenesis and related behaviours have not been previously investigated.

1.4 Current Study

While few cognitive and behavioural studies have looked at the effects of combined methamphetamine and alcohol use, previous work has found that the combination caused anxiogenic behaviour in mice which may result from cell death in the dentate gyrus (Chuang, et al 2010). If this is the case, then it could be suggested that an impairment of neurogenesis in this area could produce similar effects in addition to deficits in memory. In this study, we aimed to better understand the role that neurogenesis plays in cognitive and behavioural deficits that are induced by polydrug abuse. Moreover, we wanted to understand the dangers of polydrug use compared to monodrug use in an adolescent context using methamphetamine

and alcohol abuse. To model the long-term neurological and behavioural effects of multi-drug use in young human adults, we therefore needed to observe four groups of rats who are given different combinations of methamphetamine and alcohol and observe them in a number of apparatuses designed to measure memory and behaviour and evaluate the possible relationships between any observed deficits and levels of neurogenesis.

To this end, we examined in adolescent rats the effects of alcohol, MA, and combinations thereof, on reference memory and working memory performance, anxiety-like behaviour and hippocampal neurogenesis. To examine the persistence of the predicted behavioural deficits, rats underwent a period of non-interference following binge exposure to the drug treatments and were subsequently tested in a drug-free state. We included two tests of anxiogenic behaviour: the emergence latency/open field test and the elevated plus maze as previously described (Prut and Belzung 2003, Walf and Fyre 2007). Also, as a measure of cognition, we ran trials on a radial arm maze to assess impairments in spatial and working memory (Hodges, 1996). This enabled us to draw conclusions about the role that adult-born neurons play in cognition and emotional behaviour after polydrug exposure during adolescence.

Aims and Objectives

2.0 Aims and Objectives

- Better understand the implications of combinations of methamphetamine and alcohol use during the adolescent period.
- Investigate the role that methamphetamine, alcohol and a combination of the two have on anxiogenic-like behaviour.
- Investigate the role that methamphetamine, alcohol and a combination of the two have on cognition, specifically memory.
- Investigate the role that methamphetamine, alcohol and a combination of the two have on neurogenesis in the dorsal hippocampus, specifically the dentate gyrus.
- Examine any relationship between neurogenesis and impairments found in anxiety like behaviour
- Examine any relationship between impairments in neurogenesis and impairments of cognition.

Methodology

3. Methods

3.1 Experimental design

This study was comprised of a series of cognitive and behavioural assays and a histological analysis to determine the role that combinations of ethanol and methamphetamine exposure has on hippocampal neurogenesis and how it may alter memory and behaviour.

40 rats were assigned to one of four groups (n=10 per group) and received different combinations of drug treatments over a five-day period. The first group acted as the control group for the study and was only administered saline solutions throughout the drug treatment period. The second group was the ethanol group which received ethanol and a saline vehicle instead of methamphetamine during the treatment phase. The third group was the methamphetamine group and received a saline vehicle in the place of ethanol treatment along with a methamphetamine dose. The final group is the combined ethanol and methamphetamine group and received both of the drug treatments. Once the drug treatments were completed the rats were left undisturbed for 15 days. Afterwards all rats (n=40) were tested in a range of behavioural and cognitive assays for a period of 12 days. For the first four days the rats were tested on the emergence latency/open field test and the elevated plus maze. Over the following two days all rats are habituated in the radial arm maze before the training phase which ran for six days. The experiment runs for a total of 34 days.

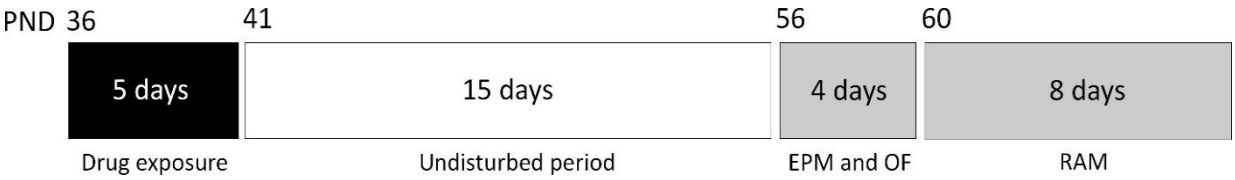

Figure 1. Drug administration protocol and behavioural testing. Rats were assigned to one of four groups (n= 9-10 per group) receiving different combinations of drug treatments (Control, ETOH, MA or ETOH-MA) over a 5-day period, starting at PND36. The rats were subsequently left undisturbed for 15 days in their home cages. A range of behavioural tests, including OF, EPM and RAM tests, were conducted between PND 56 and PND 68. On PND 69 rats were sacrificed and the brains removed for histological analysis.

3.2 Subjects

Subjects were 38, male Long Evans rats were bred and obtained from the Animal Facility of the Department of Psychology, University of Canterbury, obtained on their 28th PND. 40 rats were originally sourced for the experiment however the final batch was one animal short of what was expected and another needed to be euthanized before the beginning of the experiment. The animals were housed in pairs in polycarbonate cages (48x28x22 cm) on a reverse 12 hour light-dark cycle (lights on at 8.00pm), with standard humidity (Approx. 50%), and temperate conditions (20-22*c). The animals had food available ad libitum until PND 48 and kept at 90% of their free feeding weight until the end of the experiment. Water was available to the animals *ad libitum.* All procedures carried out were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Ethics Committee of the University of Canterbury (Protocol 2013/25R; Appendix A). Rats were allowed one week to acclimatise to their environment before any manipulations were introduced and were extensively handled during this period. The experiment began on the 36th post-natal day when the animals were approximately 5 weeks old.

3.3 Pharmacological treatments

Ethanol was procured from the University of Canterbury Animal Facilities and Methamphetamine Hydrochloride from the National Institute of Drug Abuse, NIH, USA. The animals were assigned to four separate groups, namely Control (n= 9), ETOH (n= 9), MA (n= 10) and ETOH-MA (n= 10) receiving saline, ethanol (1.5 g/kg, 20%), MA (2 mg/kg) or ethanol and MA combined (1.5 g/kg of 20% ethanol plus 2 mg/kg MA), respectively, via oral gavage. All groups (including the control group that received saline) were subject to the same handling and protocol required for the gavage procedure to avoid confounding the data from the potential stressful procedure. Dose selection for the drugs was based on studies of dose translation from animal to human (Reagan-Shaw et al., 2008) and on data from rat self-administration assays (Cotter et al., 2015; Pei et al., 2016). The volume injected remained constant for all groups (ml injected = [weight in g/160] x 1.5 for each rat). All rats were treated with a single daily dose over a five-day period. Behavioural tests began after a 15-day withdrawal period during which rats were left undisturbed in their home cages (Fig. 1). Therefore, behavioural testing occurred at a time when immature hippocampal neurons typically exhibit unique plasticity features that contribute to relevant physiological, cognitive behavioural processes (Doetsch and Hen, 2005; Ge et al., 2007).

3.4 Apparatus

3.4.1 Open field/Emergence test.

The emergence latency/open field apparatus consists of two joined compartments. The first area where the rats are initially place for the emergence latency phase of the test is a smaller dark compartment (24cm x 13cm x 45cm) with a sliding door to allow access into the larger area (900cm x 900cm x 45cm) where the open field test is conducted. The first small chamber's floor, walls and door were painted black with a perspex roof to observe the

animal. The floor of the main chamber is divided into 16 zones defined by squares by white painted lines on the dark test chamber floor.

The emergence portion of this test is used to assess anxiety or fearfulness, whereas the open field portion enables the assessment of activity and emotionality. Each rat was placed in the small, darkened start box dark side for 1 min. Then, a slide separating the box from a 900 cm x 900 cm x 45 cm open field is withdrawn to allow the rat free access to the field. The time taken for the rat to emerge into the open field is recorded (with an upper limit of 10 min). While in the open field it is noted which square the rat is located in every three sec, for 5 minutes with the slide between the field and the start box closed. This provided the data to determine whether the rat is transitioning between squares (exploratory behaviour), immobile (a discrete event during which the rat remained still for 3 seconds or more), or occupying the centre squares (comfortable in a relatively exposed position). It was also noted whether the rat is rearing up on its hind legs and at the end of the trial the number of faecal boluses left in the apparatus is counted. All observations are made via CCTV with the observer some distance from the rat being observed. Testing of all animals on this apparatus took two days.

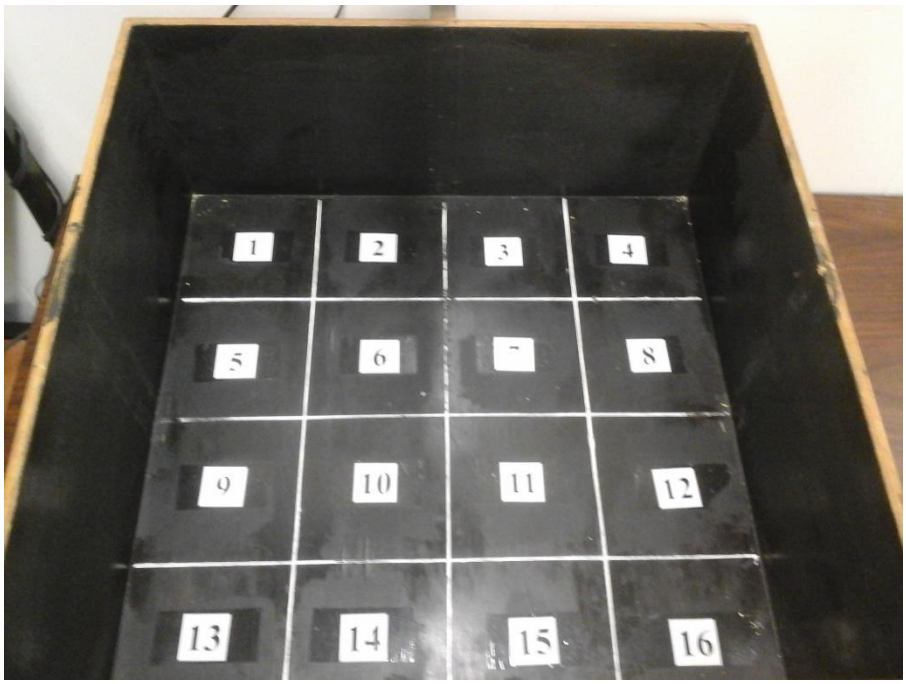

Figure 2. Image of the Open Field Maze's Arena. Each of the 16 squares have thin white borders and are numbered. 6, 7, 10 and 11 are considered centre squares. All the remaining squares are considered edges while 1, 4, 13 and 16 are also regarded as corners.

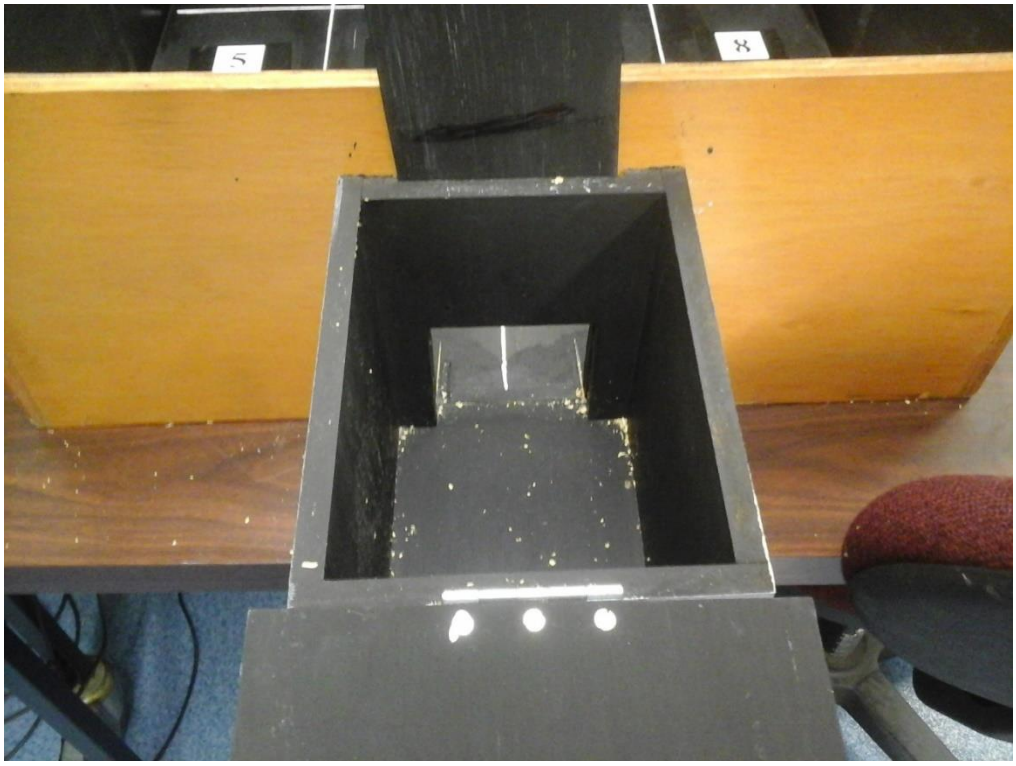

Figure 3. Image of the Emergence box. The small enclosure attached to the OFM used to house the rats before the beginning of the test. A dark small room with folding lid and sliding door to access the apparatus.

3.4.2 Elevated Plus Maze.

A 4-armed plus maze of which two arms are open (clear Perspex walls), and the other two are closed (wooden walls). The whole maze is elevated 1 meter above the floor.

This test of anxiety/fear involves placing individual rats into the centre of a 4-armed plus maze of which two arms are open (clear Perspex walls), and the other two are closed (wooden walls). The whole maze is elevated 1 meter above the floor. For 5 minutes, the rat's entries into and time spent in each arm are recorded thereby enabling calculation of preferences for entering and occupying open versus closed arms (more anxious rats prefer

closed arms as opposed to the exposed open arms). Testing of all animals on this apparatus took two days.

Figure 4. Image of the Elevated Plus Maze. Two of the four arms have transparent arms. When the test begins, the animals are placed directly in the centre of the apparatus and orientated to face a corner where two walls meet.

3.4.3 Radial Arm Maze.

After the four days of behavioural assays, rats are introduced to the radial arm maze for cognitive assays over a final eight-day period. Firstly, the rats are familiarised to the maze during a two-day period called habituation and finally training of the rats in the maze occurs over a six-day period. All observations are made via CCTV with the observer some distance from the rat being observed.

The radial maze is a useful tool to examine the neural systems that are involved in memory performance and to assess the influence of drugs on such processes (Olton 1987). The apparatus is composed of a central area that gives access to eight equally-sized arms. The arms are 70 cm long and 10 cm wide and the central area is 30 cm in diameter. In the distal extreme of each arm, a recessed cup is installed for positioning the food rewards (20 mg Noyes chocolate pellets). Underneath the recessed food cup, a hidden and inaccessible cup is also baited with the same food reward, this ensures that the animals are not rewarded for finding pellets using a scent based strategy and instead must rely on their memory of food locations. The rats are allowed to explore the maze for 10 minutes on two consecutive days in the presence of distal cues (posters and objects of different sizes) for spatial reference, which remains in place throughout training.

Training in the RAM was conducted in a similar way as described previously (Hernandez-Rabaza et al., 2010) and consisted of four blocks of six trials each, performed over six consecutive days with the need to reach a criterion of 75% accuracy. The task involves locating four pellets, each placed at the end of a different arm according to a random configuration. Configurations are specific for each rat and are kept constant throughout training. The number of spatial reference errors (reference memory [RM] errors, visits to unbaited arms) and working memory errors (working memory [WM] errors, visits to arms already visited in the same trial) is calculated and expressed as number of RM and WM errors per block. All observations are made via CCTV with the observer some distance from the rat being observed. Testing and habituation of all animals on this apparatus took a total of eight days.

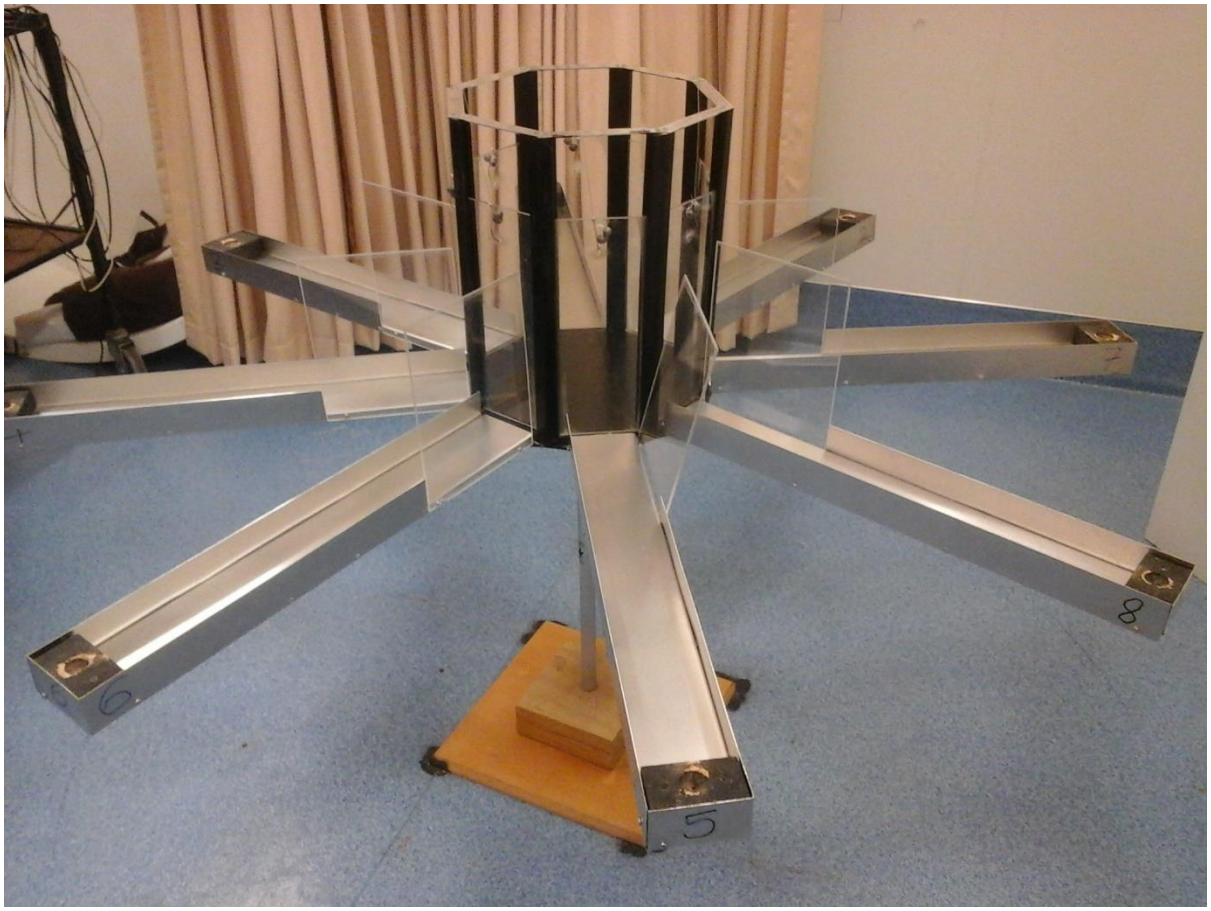

Figure 5. Image of the Radial Arm Maze. A centre platform with eight extending arms, each with a recessed feeding cup for pellets. Each feeding cup also contains a hidden and inaccessible cup which is baited to prevent the animals from relying on food scent to complete the task.

3.5 Histology and Immunohistochemistry

3.5.1 Perfusions.

After the final day of testing, the animals were perfused in order to gain the needed histology data. The animals were deeply anaesthetised with pentobarbital (125 mg/kg) and perfused transcardially with paraformaldehyde (PFA 4% in 0.1M phosphate buffer) in order to fix and remove the brain. Once the animal was heavily sedated under anaesthetic and unresponsive to pain (tested with tail-pinch technique), the thoracic cavity was opened to expose the heart. After blocking the descending aorta, a butterfly needle was inserted and secured to the left ventricle, and a herpanised saline solution was perfused. Exsanguination is achieved by making a small cut in the atrial region of the heart at the time of inserting and securing the perfusion needle into the heart. Following a saline solution, the fixative (PFA) was perfused and the head was removed. The Brains were extracted and post-fixed overnight at 4°C in the same fixative and subsequently transferred to graded saccharose solutions in 0.1M PB with 0.1% sodium azide for cryoprotection. Following perfusions, the brains were removed and 30um coronal sections through the DG (-1.6 to -6 AP from Bregma) were prepared using a microtome.

3.5.2 Doublecortin staining

Doublecortin expression was used as a sensitive marker of neurogenesis in the hippocampus (Dominguez-Escriba et al., 2006; Ferragud et al., 2010; Hernandez-Rabaza et al., 2006). Every third section (90um, 30um apart) was used to investigate DCX immunoreactivity and the immunocytochemistry was performed as previously described (Hernandez-Rabaza et al., 2006). Free-floating coronal sections were thoroughly rinsed three times with 0.1M PB and then incubated in 3% H2O2 (conc. 20%) in phosphate buffered saline with 1% Triton X-100 (PBS-Tx) for 10 minutes to quench endogenous peroxidase activity. After another rinse in PB

solution the sections were blocked with 5% Normal Horse Serum in PBS-Tx for 30 minutes to eliminate endogenous peroxidase before being rinsed again and incubated over two days in primary Goat anti-DCX (DCX, goat polyclonal anti-DCX, 1:200; Santa Cruz Biotechnology, CA, USA) antibody (1:200 dilution) with 0.1% NHS and PBS-Tx at 4°C. After incubation the sections were rinsed in PBS-Tx and incubated with Horse anti-goat IgG secondary antibody (1:400 dilution) for an hour before being rinsed again in PBS-Tx. Once rinsed the sections were incubated with biotinylated secondary antibodies and avidin-biotin horseradish-peroxidase complex (ABC kit, Vector, CA, USA) (1:200 dilution) in PBS-Tx for an hour followed by a PB rinse. DCX immunoreactivity was then visualized using 0.03% 3,3-diaminobenzidine tetrahydrochloride (DAB) with 0.01% H2O2 in a PB solution for 5 minutes after which they were rinsed thoroughly in PB to label antigenic sites. The coronal sections were then mounted from a 0.1M PB solution onto gelatine-coated slides and air dried overnight in a fume-hood. Once dried the mounted coronal sections were delipidised in gradually increasing concentrations of ethanol, first in two 70% baths of ethanol for three minutes each, 95% for five minutes, and a further five minutes in 99% ethanol. Lastly, the slides passed through two Xylene baths for five minutes each before being covered in a light layer of mounting medium (DPX) and cover-slipped. The completed slides were then air dried overnight.

3.5.3 Confocal Microscopy

Images were collected using a Nikon Eclipse Ni-E microscope and associated Nikon software. To collect data for the analysis of gaps and length of gaps in the sub granular layer of the dentate gyrus images were capture at 10x magnification with a total area of 1200um x 900um (1.33 aspect ratio). Each slide was orientated in a way to include as much of the dentate gyrus as possible beginning from the tip where the lateral and medial blade meet.

When collecting the arborisation data images were taken at 40x magnification with a total area of 300um x 225um (1.33 aspect ratio). These slides were orientated so that a thin area of the granule cell layer was present but the bulk of the image contained the molecular layer dorsal to the lateral blade or ventral to the medial blade.

For each rat, eight images were taken at approximately -2.4, -3. 3, -4.2 and -5.1mm from relative to bregma (Paxinos and Watson, 2013), at 10X, four per hemisphere, totalling 304 overall. Additional images were taken at 40X at the same locations, totalling 8 images per rat, and 304 images overall. A total of 608 distinct samples.

3.5.4 Image Analysis

Images were analysed using the UTHSCSA ImageTool software. Once the scale of the images had been calibrated into the software measuring the gaps was achieved using the distance tool, a system that measures the distance between two selected points and automatically exports the information to a table. All that remained was to move along the sub granule cell layers of the dorsal and ventral blades sampling the gaps, as this was completed it also provided the data for the total number of gaps. A gap was defined as a distance between two DCX+ cells greater than 10-μm along the subgranular layer of the DG. This criterion was chosen because although some minute gaps are expected as part of morphological oddities in the DG we only wanted to observe the pronounced gaps in subgranular connectivity which could be occupied by one or more cells. For this reason, 10μm was selected as it is the approximate size of the soma of granule cells in the dentate gyrus (Tatsunori et al., 2011).

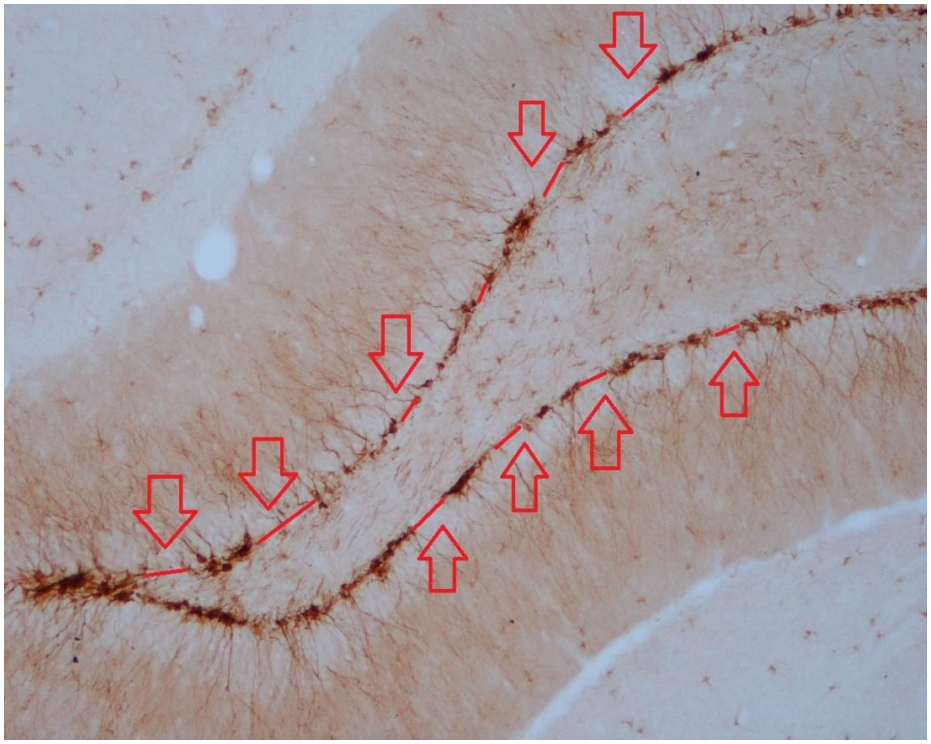

Figure 6. Image of the dentate gyrus at 10x magnification. An example of the process of sampling the length and total number of gaps along the lateral and medial blades as defined by the image analysis protocol.

After recalibrating the scale for the 40x images, arborisation data was collected by observing the extent that axons and their dendrites were present in the molecular layers of the lateral and medial blades. First the images were converted to greyscale and then processed using sobels edge detector which emphasised the edges within the image. As the labelled dendrites and axons are so thin this process effectively separates them from the background noise found within the image in a more binary way. Next we selected the area we wanted to examine using the free-select tool ensuring that we excluded the granule cell layer so that only the arborisation into the molecular layers are processed. Finally, the selected area is analysed using histogram by colour and the brightest (or detected edges of the cells) is given as a value out of 100, giving us an estimate of the total DCX+ arborisation in the selected area as a percentage.

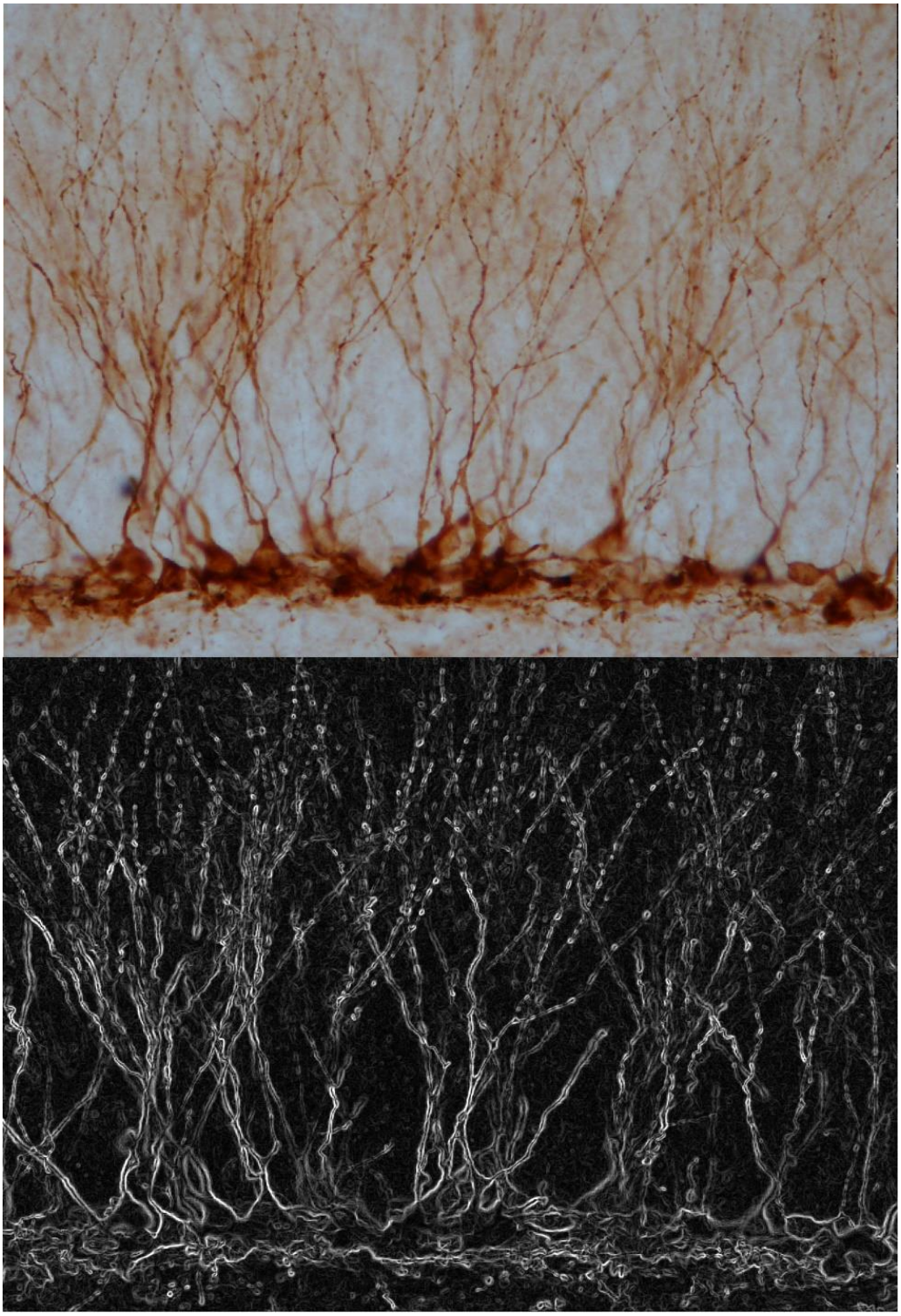

Figure 7. Image of the molecular layers of the dentate gyrus at 40x magnification. An example of the process required to investigate the extent of arborisation along the lateral and medial molecular layers as defined by the image analysis protocol. Only the area of arborisation extending outwards from the somas of the cells in the granule cell layer was analysed.

Results

4. Results

4.1 Statistical analysis

The data was analysed by means of factorial or repeated measures analysis of variance (ANOVA), as required. Significant main or interaction effects were further explored with Newman-Keuls (N-K) tests. To explore relationships between indices of adult hippocampal neurogenesis and behavioural measures, correlation coefficients were calculated based on the Pearson's correlation test. Significant levels were set at $\alpha = 0.05$ for all experiments and measures.

4.2 Behavioural test results

Emergence and open field test. The latency to emerge from the start box into the open field was not significantly affected by the drug treatments. ANOVA indicated no effect of the factor, Treatment, on latency [$F_{3,34} = 1.319$, $p = 0.284$] (data not shown). The number of transitions, a measure of locomotor activity in the OF, was significantly reduced by MA and ETOH-MA treatments, as shown by the ANOVA [$F_{3,34} = 4.966$, $p = 0.006$] and the N-K *post hoc* tests (Fig. 8a), as was immobility time [$F_{3,34} = 1.598$, $p = 0.208$] (Fig. 8b), whereas rearing [$F_{3,34} = 1.598$, $p = 0.208$] and defecation [$F_{3,34} = 1.782$, $p = 0.169$] (data not shown) showed no interaction. However, the time spent in the centre of the arena, a measure of fearfulness and emotionality, was significantly reduced by prior exposure to ETOH, MA or ETOH-MA, as indicated by ANOVA [$F_{3,34} = 4.738$, $p = 0.007$] and N-K means comparisons (Fig. 8c).

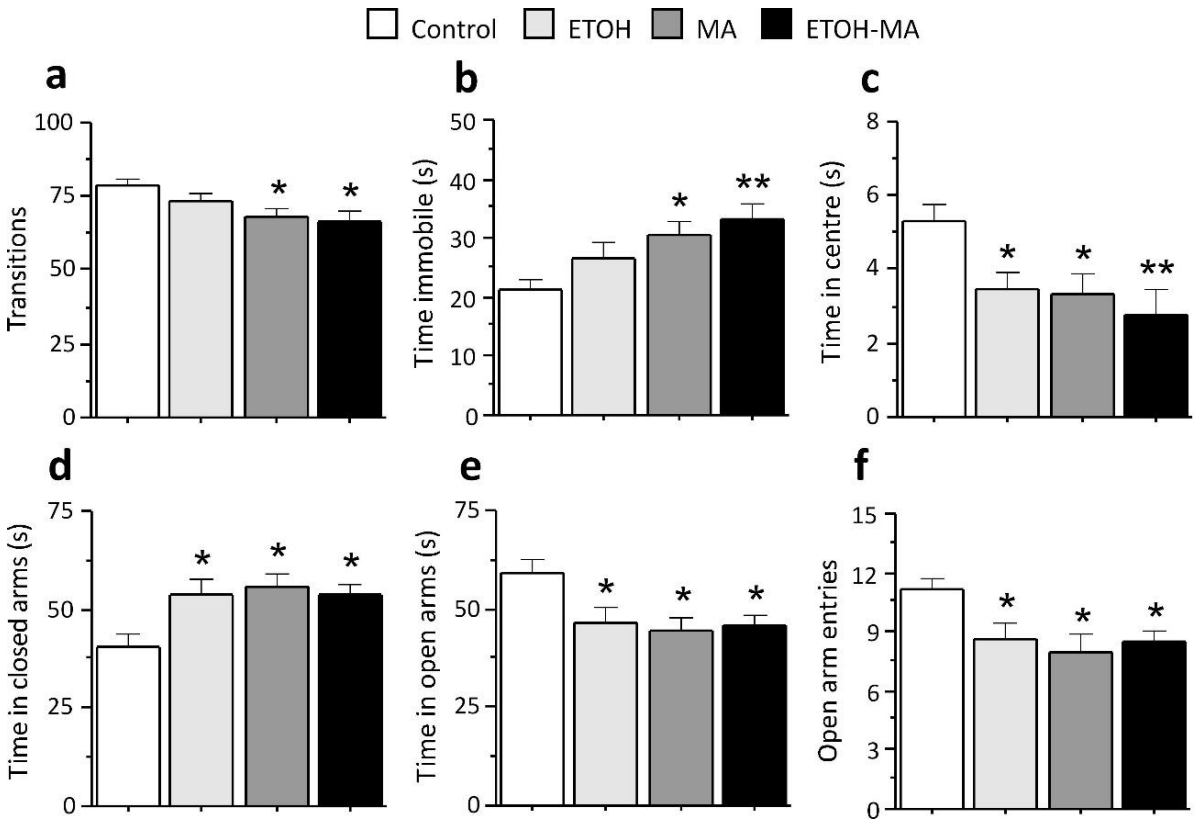

Figure 8. Behavioural effects induced by alcohol and MA exposure in the OF and EPM tests. In the open field maze, rats treated with MA or ETOH-MA transitioned between squares less frequently and exhibited greater periods of total immobility **(a, b)**. While all treatment groups avoided the centre of the maze compared to the control, only the ETOH-MA group spent significantly less time occupying it **(c)**. In the EPM, all of the drug treatment groups spent less time in open arms, more time in closed arms, and exhibited fewer open arm entries **(d, e, f)**.

Elevated Plus Maze. In the EPM test, all three drug treatment groups displayed similar behaviour, characterised by significantly increased time in the closed arms [$F_{3,34}$= 4.762, p= 0.007], reduced time in the open arms [$F_{3,34}$= 4.738, p= 0.007], and decreased number of entries into the open arms [$F_{3,34}$= 3.348, p= 0.030], thus demonstrating augmented anxiety-like behaviour (Fig. 8d-8f).

4.3 Tests of memory function

Radial Arm Maze. The results of the memory tests conducted in the RAM indicated that ETOH, MA and ETOH-MA exposure produced modest, but significant impairments in reference memory. Repeated measures ANOVA was performed for RMEs, yielding a significant effect of the Treatment factor [$F_{3,34}$= 10.919, p< 0.001], a significant effect of Block [$F_{3,102}$= 406.801, p< 0.001], which indicated that learning occurred across the blocks of trials, but not an interaction Treatment x Block [$F_{9,102}$= 0.919, p= 0.512]. N-K tests showed that all three experimental groups differed from control values (Fig. 9a). An ANOVA with repeated measures was also computed to analyse WMEs in the RAM. There was a significant effect of Treatment [$F_{3,34}$= 5.569, p= 0.003] and a significant effect of the Block factor [$F_{9,102}$= 143.471, p< 0.001], again denoting learning across blocks of trials (Fig. 9c). No interaction Treatment x Block was found [$F_{9,102}$= 0.568, p= 0.745]. Inspection of the main effect of Treatment with N-K tests revealed that only the group receiving ETOH-MA exhibited significant impairments in working memory performance, compared to the control group.

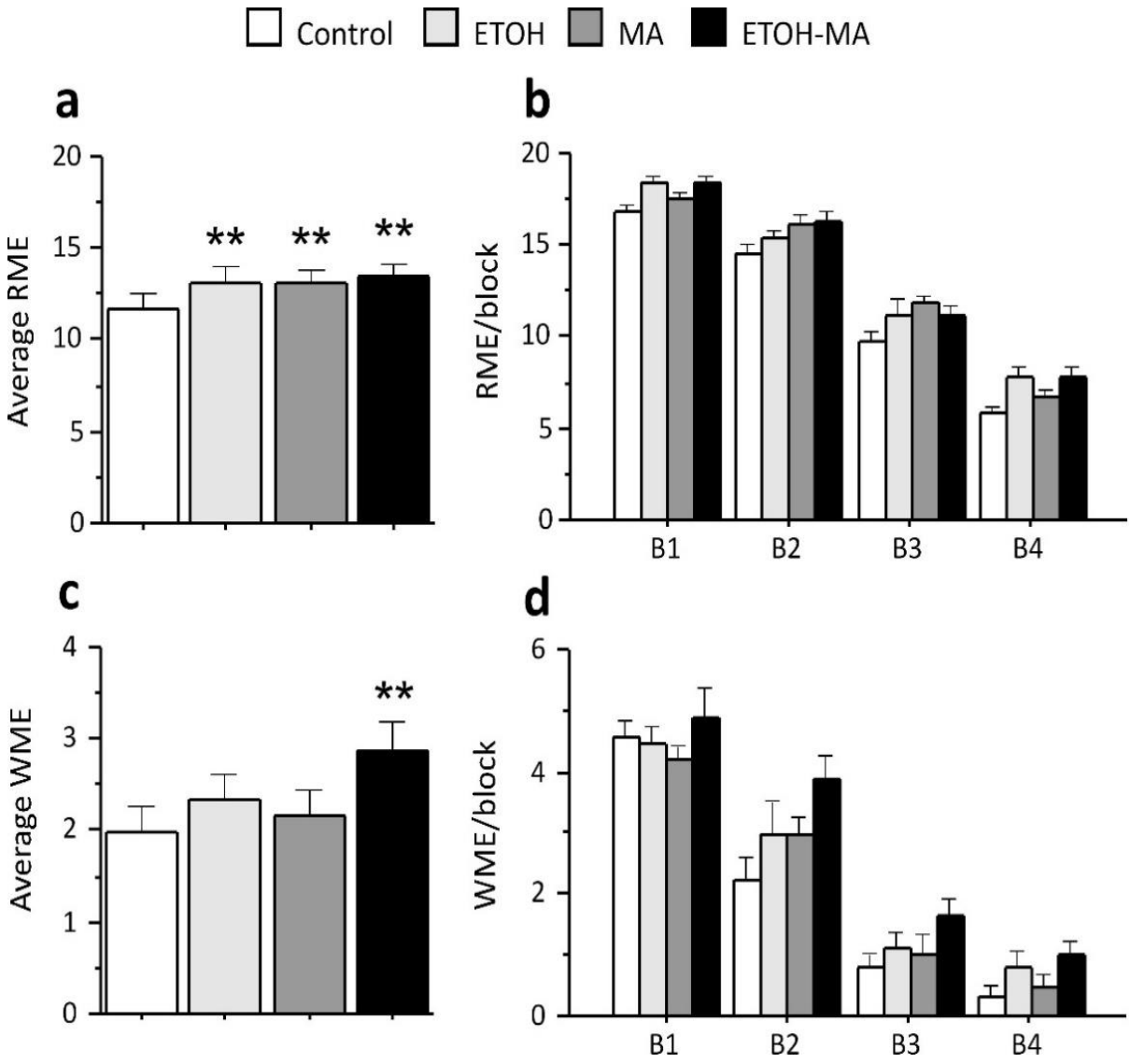

Figure 9. Memory deficits produced by alcohol and MA treatments in the RAM. Rats in all three drug treatment groups exhibited a significantly higher number of RMEs across the blocks of trials compared to the control group **(a, b)**. However, only the rats in the combined ETOH-MA group produced significantly more WMEs than their control counterparts **(c, d).**

4.4 Neurogenesis

Adult hippocampal neurogenesis. To analyse the number of DCX-void gaps in the DG, one-way ANOVA was calculated with between-factor, Treatment. ANOVA showed a significant effect of Treatment [$F_{3,34}$= 3.003, p= 0.044]. The number of DCX-void gaps in the DG was significantly increased only by the combined treatment with ETOH and MA, as shown by

post hoc tests (Fig. 10a and Fig 10d-10g). The length of gaps was analysed using the same ANOVA procedure. ANOVA yielded a significant effect of the Treatment [$F_{3,34}$= 10.171, p< 0.001]. *Post hoc* analysis of the main factor, Treatment, showed that rats treated with MA alone or the ETOH-MA combination displayed larger DCX-void gaps in the DG (Fig. 10b). Albeit the pattern of results appeared to be similar to that observed for gaps and lengths of gaps, ANOVA indicated no effect of the Treatment factor [$F_{3,34}$= 1.138, p= 0.348] for the arborisation data (Fig. 10c), which could be accounted for by the slightly greater variability of the arborisation measures.

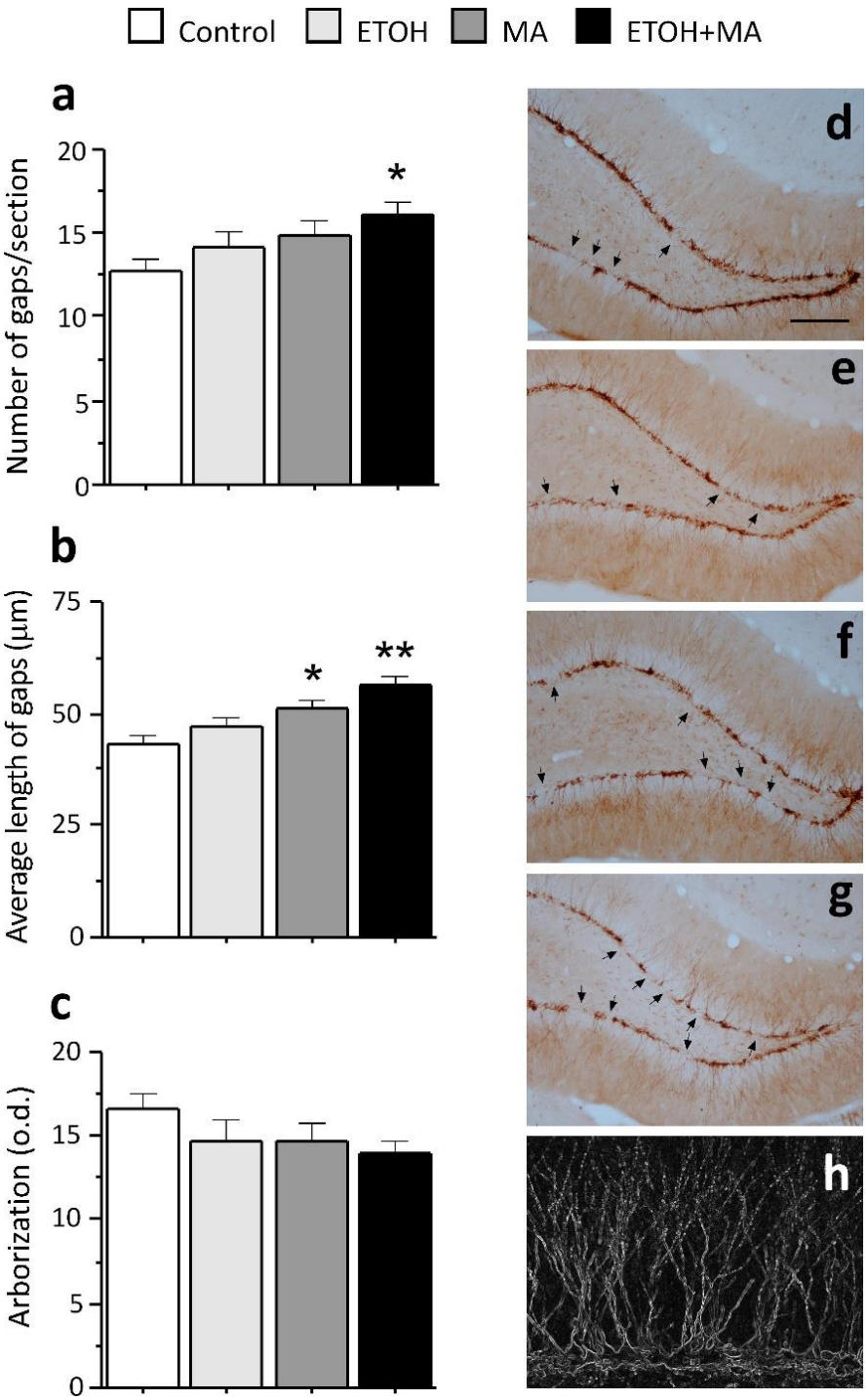

Figure 10. Alterations in adult hippocampal neurogenesis after alcohol and MA exposure. Rats treated with the ETOH-MA combination displayed a significantly greater number of DCX-void gaps in the subgranular layer of the DG **(a)**. Rats in both the MA and ETOH-MA treatment groups showed a significant increase in the average length of DCX-void gaps **(b)**. The levels of arborisation of arborisation of DCX+ neurons did not differ significantly across treatment groups **(c)**. Representative photomicrographs show the presence DCX-void gaps in the DG of rats receiving saline **(d)**, ethanol **(e)**, MA **(f)** or ETOH-MA **(g)** treatments. Image in **(h)** shows the rendering of dentritic ramifications of DCX+ neurons after image processing and prior to calculation of optical density.

To examine the relationship between indices of DCX expression and the significant behavioural outcomes, we calculated correlation coefficients between pairs of variables

*, p< 0.05; **, p< 0.0	OF			EPM			RAM	
	Transitions	Immobility	Time centre	Time open	Open arm	Time closed	RME	WME
Number gaps	-0.38*	0.39*	-0.22	-0.01	-0.25	0.01	0.28	0.37*
Gap length	-0.48**	0.44**	-0.43**	0.10	0.10	-0.10	0.16	0.07
Arborisation	0.31	-0.29	0.27	-0.09	-0.07	0.09	0.07	-0.07

Table 1. Correlation coefficients between pairs of behavioural measures and indices of adult hippocampal neurogenesis.

The results showed significant correlations between the numbers of DCX-void gaps with locomotor transitions and immobility in the OF test ($p < 0.01$ in both cases), and with working memory performance in the RAM ($p < 0.05$). On the other hand, the length of gaps was correlated with all three behavioural measures in the OF task ($p < 0.01$ in all three cases).

Discussion

5. Discussion

The current experiments provided a straightforward demonstration that relatively brief exposure to alcohol and MA, even at moderate doses, impacts negatively and persistently on cognitive performance, emotional behaviour and hippocampal plasticity. The data showed that exposure to alcohol, MA or an alcohol and MA combination treatment resulted in a range of behavioural, emotional and cognitive impairments that were apparent even after a prolonged period of withdrawal. The expression of such deficits was mirrored by maladaptive changes in adult hippocampal neurogenesis. We identified specific correlations with neural markers of neurogenesis for some of the behavioural processes affected by the drug treatments. Moreover, the current study uncovered some of the negative effects associated with the concurrent administration of alcohol and MA, which by and large exacerbated the neurological and behavioural alterations induced by either drug administered separately, and in some cases caused new ones to emerge.

MA is an addictive substance that exerts potent stimulant and euphoric effects by promoting the vesicular release of dopamine (DA) and reversing the transport of DA through the DA transporter at central synapses (Fleckenstein et al. 2007). Abusers of MA typically administer the drug in "runs" lasting several days in a binge-like pattern interspersed with periods of withdrawal (Haile et al. 2009). Epidemiological studies have shown that the concurrent use of alcohol is prevalent amongst MA users (Parsons et al. 2007). A study conducted in the city of New York reported that more than 60% of regular MA users consumed alcohol simultaneously (Halkitis et al. 2005), and more recently an association between stimulant, but not cannabis, intoxication and binge drinking has been reported amongst young adults (McKetin et al. 2014). In spite of this evidence, few studies have investigated the potentially hazardous interactions between alcohol and MA. A comprehensive study conducted by Kirkpatrick and collaborators recently examined the

physiological, psychomotor and cognitive effects induced by the co-administration of alcohol and MA in humans. The results showed that the combination increased cardiovascular responses and feelings of euphoria, supporting previous observations (Mendelson et al. 1995). Furthermore, MA reduced alcohol-specific self-ratings of intoxication and alcohol-induced cognitive and psychomotor performance impairments, whilst alcohol decreased MA-associated sleep disturbances (Kirkpatrick et al. 2012). In rats, N-demethylation and *p*-hydroxylation of the aromatic ring of MA may be inhibited in the presence of alcohol, leading to impaired MA metabolism and protracted behavioural effects (Yamada et al. 2001). Similarly, in humans treated with either MA alone or alcohol combined with MA, differences in the relative proportions of *p*-hydroxylated metabolites to unchanged MA in urine prompted the suggestion that alcohol leads to suppression of MA metabolism (Shimosato 1988). These findings suggest that alcohol may potentiate some of the physiological actions of MA and prolong its psychoactive, and potentially neurotoxic, effects.

In the current study, animals exposed to alcohol, MA or the two drug treatments combined displayed altered exploratory behaviour and emotionality in the OF and EPM tests long after treatment cessation. Both the OF and the EPM are popular tests of unconditioned anxiety in rodents but their validity in providing unequivocal measures of anxiety has been questioned on several grounds (Ennaceur 2014). Although measures of emotionality (i.e., latency to emerge, grooming, defecation) were not affected by the treatments in the OF task, both tests revealed strong alterations, with marginally greater effects of the combination treatment in the OF test. However, while some markers of neurogenesis (i.e. number of DCX-void gaps and length of gaps) correlated with exploratory behaviour in the OF test, no such correlations were observed for anxiety-like behaviours in the EPM, suggesting that the observed effects in these two tasks may relate to different behavioural constructs. We do recognize that this apparent discrepancy requires careful consideration. Behavioural, anatomical and gene

expression studies support functional segregation within the hippocampus, with the "cold" dorsal domain being primarily involved in cognition and spatial navigation, by way of fornix projections to the medial and lateral mammillary nuclei and the anterior thalamic complex, and in motivated exploratory behaviour, through inputs to the nucleus accumbens and caudoputamen (Fanselow and Dong 2010). In turn, the ventral hippocampus can be thought of as a "hot" domain, as it relates to stress, emotion and affect (Fanselow and Dong 2010), contributing critically to the neural systems involved in behavioural inhibition in situations of approach-avoidance conflict (Schumacher et al. 2016), which are not necessarily represented in the OF and EPM tasks (Ennaceur 2014). Moreover, lesions of the ventral hippocampus produced anxiolytic-like effects in the EPM (Kjelstrup et al. 2002), as did transient inactivation with the GABA-A agonist, muscimol, while inactivation of the dorsal hippocampus elicited anxiogenic-like behaviour (Zhang et al. 2014), suggesting that the dorsal and ventral regions subserve different aspects of EPM-related behaviour. Albeit neurogenesis in the adult hippocampus has been implicated in the neurobiology of depression and anti-depressant action, neurogenesis in the ventral dentate gyrus may be preferentially involved in emotions and affective regulation (Sahay and Hen 2007; Tanti and Belzung 2013). Moreover, the vast majority of studies published to date lend support to the notion that adult hippocampal neurogenesis decisively influences stress and affective behaviour, but not anxiety. For example, suppression of neurogenesis after focal ionizing irradiation, antimitotic treatment or genetic manipulations did not affect anxiety-like behaviour in a variety of paradigms, including novelty suppression of feeding (David et al. 2009; Surget et al. 2008), OF (Fuss et al. 2010; Schloesser et al. 2010) and EPM tests (Saxe et al. 2006; Snyder et al. 2011). Taken together, these findings suggest that the deficits we observed in the EPM and OF tests following drug exposure may have been independent of the neurogenic alterations in the dorsal DG. The correlations we noted between activity measures in the OF test and

hippocampal neurogenesis are more likely to reflect an association with general deficits in exploratory activity, which are known to parallel neurogenic alterations after drug exposure (Canales and Ferrer-Donato 2014). However, additional studies are required to investigate this question further.

The present study demonstrated that exposure to alcohol, MA and combined alcohol and MA produced long-term memory deficits. The mnemonic alterations produced by alcohol and amphetamine-like substances in humans and animals are well documented (Canales 2010; Fernandez-Serrano et al. 2011). The current data showed that all three drug treatments increased RMEs to the same extent during the acquisition of the RAM task. However, these deficits did not correlate with neurogenic alterations, which were only present in rats treated with MA or ETOH-MA. This is consistent with the observation that inhibition of adult hippocampal neurogenesis does not generally impact on this type of memory (Hernandez-Rabaza et al. 2009; Wojtowicz et al. 2008), except when the long-term retention of a spatial location is required (Jessberger et al. 2009). Interestingly, the present results revealed that only the treatment with MA and alcohol combined had deleterious effects on working memory function, and that WMEs correlated with the presence of DCX-void gaps in the DG. The dorsal hippocampus, specifically the DG, is strongly involved in spatial working memory (Hernandez-Rabaza et al. 2007; Lee and Kesner 2003), whereas the involvement of adult neurogenesis in this function remains controversial. Contrasting findings have been reported depending on sex, task, training protocols, retention intervals and type of analysis of neurogenic markers (Hernandez-Rabaza et al. 2009; Hillerer et al. 2013; Recinto et al. 2012; Winocur et al. 2006). Albeit the association between adult neurogenesis and working memory remains contentious, combinations of alcohol and MDMA have been previously reported to produce concurrent cognitive and neurogenic deficits (Canales and Ferrer-Donato 2014; Hernandez-Rabaza et al. 2010).

One interesting observation was that WMEs correlated with the number of DCX-void gaps in the DG but not with the average length of the gaps, suggesting that the presence of multiple discontinuations along the subgranular layer of the DG may have disrupted information processing for items that are spaced closely in time, as required in within-trial learning of the kind required in the RAM. Memory-related activity in the DG is thought to depend on the ability of granular neurons to generate sparse representations of inputs (Petrantonakis and Poirazi 2015; Piatti et al. 2013), and therefore the presence of multiple neuronal gaps could make it more likely that relevant inputs from relevant areas are "missed". It is also worth noting that the arborisation of adult-generated neurons was not significantly affected by the drug treatments, suggesting that exposure to MA or ETOH-MA affected the survival, but not the maturation, of neural precursors. Accordingly, arborisation indices did not correlate with any of the behavioural changes observed.

Conclusions

6. Conclusions

In conclusion, the current study demonstrated that the concurrent administration of alcohol and MA produces long-lived cognitive, emotional and neurogenic deficits that are generally greater than those observed following separate administration of these substances. These findings emphasize the potential mental health risks associated with mixing alcohol and MA during several consumption episodes.

Poly-drug abuse is a more critical and relevant example of drug abuse not only because it is a more realistic model of substance use but also because it shows the compounding effects on behaviour and brain development that both contribute to wearing down a person resilience to addiction. Negatively impacting on anxiety-like behaviour and hippocampal neurogenesis can impair a drug users process of recovery and may make them more prone to succumbing to addiction in the future, further drawing the users down into the spiral of drug addiction and substance abuse.

Future research should be focused on different combinations of drugs used in a poly-drug abuse situation, specifically the relationship alcohol plays with more common illegal substances like marijuana, legal prescription medications, ‘grey area’ party-pills and incorporate other markers of neurogenesis like BrdU to compare alongside doublecortin.

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Appendices

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz
Ref: 2013/25R

8 November 2013

David Loxton

Department of Psychology UNIVERSITY OF CANTERBURY

Dear David

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Memory and behavioural deficits induced by combined ethanol and methamphetamine exposure in adolescent rats: role of adult neurogenesis"

Approval has been granted:

(a) for the use of 40 Long-Evans Rats

(b) for your research project to be undertaken from 4 November to 31 March 2013. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MAF statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MAF Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, whichever comes first. If no animals have been manipulated in your project please provide a "Nil" return.

Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

James Briskie

Associate Professor Jim Briskie
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